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Potentiating role of copper on spatial memory deficit induced by beta amyloid and evaluation of mitochondrial function markers in the hippocampus of rats

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Mounting evidence suggests that copper, a crucial element in normal brain function, plays an important role in the etiology of Alzheimer's disease, which is known as a neurodegenerative mitochondrial disorder. However, the precise mechanisms of its effects on cognitive and mitochondrial functions through the CNS have not been thoroughly recognized yet. In this study, we aimed to investigate the long-term (3-week) effects of copper sulfate (50, 100 and 200 mg kg^{-1} day^{-1}) exposure on learning and memory as well as on mitochondrial function in the hippocampus of rats in the presence and absence of beta amyloid (1 μg μl^{-1} per side) intrahippocampally (IH). After three weeks of copper exposure through drinking water, acquisition and retention of spatial memory were measured by the Morris water maze (MWM) test. Various parameters of mitochondrial function were also evaluated. Our data show that copper damaged the spatial learning and memory and also exacerbated the memory deficit induced by Aβ injection in rats in a dose-dependent manner. Mitochondria isolated from the hippocampus of rats treated with copper showed significant increases in ROS formation, mitochondrial swelling, lipid peroxidation, glutathione oxidation, outer membrane damage, and collapse of MMP, decreased cytochrome *c* oxidase activity, and finally increased ADP/ATP ratios. Our results indicate that copper overloading in the hippocampus of rats causes mitochondrial dysfunction and subsequent oxidative stress leading to cognitive impairment. This study also reveals that copper can potentiate Aβ deleterious effects on spatial memory and brain mitochondrial function.

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1. Introduction

Copper (Cu^{2+}), an essential trace element, is an integral part of many important enzymes required for many vital biological processes such as energy production, scavenging of free radicals, iron mobilization, neurotransmission and catalytic functions in living cells and their organelles, particularly mitochondria.[1] Of all the metals causing pollution, copper is a common pollutant in aquatic environments, especially those which are surrounded by populating areas with increased discharge through various industrial and domestic effluents.[2] Therefore, Cu^{2+} toxicity has been linked to chronic Cu^{2+} overload and/or exposure to excess copper caused by accidents, occupational hazards, environmental contamination or inborn copper metabolic disorders.[1,3] Besides that, a relatively high level of this redox active metal can be toxic and can mediate the formation of reactive oxygen species (ROS) in aerobic cells.[4,5] Homeostatic alterations in cerebral copper levels have also been implicated in the pathogenesis of Alzheimer's disease (AD) and possibly other neurodegenerative disorders such as Parkinson's and Prion diseases.[6,7]

Alzheimer's disease (AD) is an epidemic type of dementia in the U.S. and other developed countries. It has been evaluated that 10% of people in their 60s, 20% in their 70s, and 30% in their 80s have AD in the U.S.[8,9] Gradually, our population is growing older, so we have more AD, a disease of aging. Some hypothesize that the epidemics of AD in populations over the age of 50 are mainly due to inorganic copper ingestion in drinking water and copper supplements.[10]

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Amyloid beta (Aβ), the main component of senile plaques in Alzheimer's disease is generated from amyloid precursor protein (APP) cleavage by beta-site APP-cleaving enzyme 1 (BACE1).[11,12] Accumulating evidence suggests that Cu^{2+} can promote Aβ aggregation and reduce Aβ degradation in a dose-dependent manner.[13] The mRNA and protein expression levels of APP and BACE1, which play an important role in Aβ generation, were increased in cells exposed to Cu^{2+}.[11,14,15] Some studies suggest that the NEP, a protease that degrades Aβ expression levels in mRNA and protein was also decreased in cells treated with Cu^{2+}.[16,17] Two main receptors, the low-density lipoprotein receptor-related protein 1 (LRP1) and advanced glycation end products (RAGE) on the BBB, play an important role in controlling Aβ levels in ISF. LRP1 is a primary transporter of Aβ across the BBB out of the brain, while RAGE is a main transporter protein for Aβ influx in ISF.[18,19] Brain endothelial mRNA expression of LRP1 was found to be lower in the brain tissue from AD patients than controls, leading to Aβ accumulation in the ISF.[20–23] Some have suggested that Cu^{2+} in drinking water leads to a decrease of Aβ clearance in the brain.[23,24]

There are two pools of copper in the blood, one which is "safe" copper, covalently bound to ceruloplasmin (cp), and the other, called "free" copper, loosely bound to albumin and other small molecules in the blood.[25] The free copper, which makes up about 5–15% of the total serum copper is the potentially toxic form. For example, in Wilson's disease, an inherited disease of copper accumulation, the free copper pool is greatly expanded.[26]

It turns out, as reported by Squitti's group in Italy, that there are elevated free copper levels in AD patients and they have also shown that free copper levels negatively correlate with cognition in AD patients.[25,27]

Animal model studies have provided a powerful piece of evidence linking AD disease to copper toxicity. Sparks *et al.*[23] observed that adding just 0.12 ppm (0.75 μM) copper (one-tenth of the environmental protection agency human consumption limits) to drinking water was sufficient to precipitate the accumulation of Aβ in the brains of cholesterol-fed rabbits. Subsequent studies indicated that adding Cu^{2+} to the drinking water of Cholesterol-fed rabbits produced an 80% deficit in their ability to acquire complex memory and learning compared to those who were fed the same diet without adding copper in the drinking water.[23]

Previous studies reported that Cu^{2+} with high affinity binds soluble monomeric Aβ *via* binding sites in the N-terminus, and coordinates with Aβ in a planar configuration with histidines (His6, His13, and His14)[28,29] and Tyr10.[30] Actually, Cu–Aβ interactions can increase free radical generation and neurotoxicity. When Aβ binds free ionic copper in the presence of appropriate reductants such as cholesterol, ascorbic acid or dopamine, the Aβ–Cu(II) complex can continue to generate significant quantities of ROS by catalyzing the reduction of Cu^{2+} to Cu^{1+}.[28,31]

During the energy production in mitochondria, ROS is produced as a byproduct of oxidative phosphorylation. Thus, the mitochondrion is supposed to be one of the important sources of ROS production.[32,33] Increased ROS production caused mitochondrial and cellular dysfunction. A negative correlation has been found between oxidative stress, mitochondrial dysfunction and impairments in behavioral tests.[34] Recent studies have shown that copper administration caused a reduction in the activities of mitochondrial enzymes and induction of the mitochondrial permeability transition (MPT). The MPT is determined by the opening of the permeability transition pore (PTP), which is located in the inner mitochondrial membrane (IMM). This increased permeability of IMM led to a dissipation of the mitochondrial membrane potential and reduced ATP production in the rat brain.[35,36] It has been suggested that mitochondrial respiration plays a vital role in the plasticity needed for spatial learning and memory.[37] It can be hypothesized that copper results in spatial learning and memory impairments through induction of mitochondrial dysfunction in the hippocampus. However, this hypothesis needs direct confirmation because far too little consideration has been paid to the mechanistic relation between mitochondrial dysfunction and learning and memory impairments caused by copper alone and in combination with Aβ.

Based on these reports and observations, we aimed to evaluate the effects of different doses of copper sulfate in drinking water in the presence and absence of Aβ on spatial memory retention in adult male rats using the Morris Water Maze (MWM) task. We also investigated the possible mitochondrial mechanisms mediating the effects of copper ion solely and with Aβ on spatial learning and memory in isolated brain hippocampi of rats.

2. Materials and methods

2.1. Materials

$CuSo_4$ was purchased from Merck (Darmstadt, Germany). Amyloid beta (1–42), 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), rotenone (Rot), dimethyl sulfoxide (DMSO), D-mannitol, thiobarbutiric-acid (TBA), 2′,7′-dichlorofluoresceindiacetate (DCFH-DA), Tris–HCl, sodium-succinate, sucrose, KCl, Na_2HPO_4, $MgCl_2$, Rhodamine123 (Rh123), Coomassieblue, Ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid(EGTA), xylazine and Ketamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used for this experiment were of the best analytical and pharmaceutical grade available.

2.2. Animals

Male rats of the Albino-Wistar strain weighing 220–250 g were obtained from the Faculty of Pharmacy, Tehran University of Medical Sciences. All animal groups were housed in cages until surgery and handled daily with free access to food and water. The light/dark cycle was maintained at 12 h/12 h and temperature and humidity were controlled. All animal manipulations were approved by the Tehran University of Medical Sciences Animal Ethics Committee. Efforts were made to reduce the number of animals used and to minimize animal suffering.

2.3. Preparation of beta-amyloid peptide 1–42 (Aβ 1–42)

The Amyloid beta 1–42 was dissolved in phosphate buffer saline (PBS 0.1 M) with a concentration of 200 μg μl^{-1} and incubated

for 5 days at 37 °C. Aliquots of Amyloid beta were stored at −20 °C until they were used. On the test day, they were diluted to reach a final concentration of 1 μg μl^{-1}.

2.4. Stereotaxic surgery

Rat anesthetization was performed by intra-peritoneal injection of 100 mg kg^{-1} Ketamine hydrochloride combined with 20 mg kg^{-1} xylazine before placement in a stereotaxic device (Stoelting, USA). An incision was made along the midline. The surgical area was cleaned and in order to decrease bleeding, a drop of dental hemostop was employed. Intrahippocampal injections of amyloid beta at the CA1 site were made 3 mm posterior and 2.1 mm lateral to bregma and 2.2 mm ventral to the surface of the skull, according to Paxinos and Watson's atlas (Paxinos and Watson, 2006). Amyloid beta or vehicle (1 μl per site) was infused bilaterally into the CA1 area (0.5 μl min^{-1}). All microinjections were performed with a 10 μl Hamilton syringe and the needle was left in the tissue for 1 min after each injection.

2.5. Experimental design

In the present study, the animals were divided into the following groups (n = 7 for each group): (i) a vehicle group, which received bilateral intra-CA1 administration of PBS (the vehicle for Aβ) (1 μl per side); (ii) an Aβ-injected group, which received Aβ (1 μg μl^{-1} per side) bilateral intra-CA1; (iii) a control group, which only received tap water; (iv) copper groups, which received 50, 100 and 200 mg kg^{-1} Cu^{2+} daily in the drinking water for 21 days; (v) combination groups, which first received bilateral intra-hippocampal infusions of Aβ (1 μg μl^{-1} per side) and then received copper (50, 100 and 200 mg kg^{-1} daily in the drinking water for 21 days); and (vi) a positive control group for behavioral tests, which received 1 μg per side nicotine intrahippocampally. The aforementioned groups entered two experimental protocols: behavioral experiments and mitochondrial studies.

2.6. Spatial learning and memory assessment

The spatial memory of all the experimental groups was evaluated by the Morris water maze (MWM) test. This maze included a black circular pool with a diameter of about 136 cm and a depth of 35 cm. The pool was filled with water to a depth of 40 cm (25 ± 2 °C). This pool was divided into four equal quadrants and an invisible platform (10 cm in diameter) was located 1 cm under the water surface in the center of the north-west quadrant (target quadrant). The rats were trained for 4 days such that each day included one block consisting of four trials. In each trial, the rats were randomly placed in one of the four quadrants. A video camera was located just above the pool, linked to a computer and equipped with Ethovision software (Noldus Information Technology, Wageningen, Netherlands) that recorded the swimming pathway and related data. Animals were allowed to swim in the pool for a maximum of 90 seconds. If the rats did not find the hidden platform within this period, the researcher manually guided the animal to the platform. The rats were then rested on the platform for 20 s. Learning capabilities were measured by quantitative computer data in terms of escape latency (time to find the platform), traveled distance (path length to reach the platform), and swimming-speed. After four training days, the rats were tested for probe trial. In the probe trial test, the platform in the target quadrant was removed. The rats were released on the opposite site of the target quadrant and allowed to swim for 90 seconds freely. The time each animal spent in the target quadrant was measured in the probe test.

2.7. Hippocampal mitochondrial function

2.7.1. Hippocampal mitochondrial isolation. After completion of MWM, all animals were sacrificed by cervical decapitation and the brain hippocampal tissues were obtained. Then, with a glass handheld homogenizer, these tissues were minced and homogenized. Mitochondria were prepared from the rats' hippocampi by differential centrifugation.[38] First, the samples were centrifuged (at 1500g, 10 min at 4 °C) and the broken cell debris and nuclei were sedimented. The supernatant was exposed to centrifugation again at 10 000 × g for 10 min. The upper layer was discarded and the mitochondrial pellet was washed and suspended in the isolation medium and centrifuged again (at 10 000g, 10 min). Final suspensions of mitochondrial pellets were prepared in Tris buffer containing (0.05 M Tris–HCl, 0.25 M sucrose, 2.0 mM $MgCl_2$, 20 mM KCl and 1.0 mM Na_2HPO_4, pH of 7.4) at 4 °C, except for mitochondrial samples used to measure the ROS level, mitochondrial membrane potential (MMP) and swelling, which were incubated in respiration buffer (10 mM Tris, 0.32 mM sucrose, 20 mM Mops, 0.5 mM $MgCl_2$, 50 μM EGTA, 5 mM sodium succinate and 0.1 mM KH_2PO_4), MMP assay buffer (68 mM D-mannitol, 220 mM sucrose, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM sodium succinate, 50 μM EGTA, 10 mM HEPES and 2 μM rotenone) and swelling buffer (3 mM HEPES, 70 mM sucrose, 230 mM mannitol, 5 mM succinate, 2 mM Tris-phosphate and 1 μM of rotenone), respectively. Protein concentrations were measured using the Coomassie blue protein-binding protocol as explained by Bradford.[38] For the normalization process in all the following assays, the mitochondrial samples (0.5 mg mitochondrial protein per ml) were used. To guarantee high quality mitochondrial preparation, all the steps were operated on ice.

2.7.2. Measurement of the hippocampal mitochondrial ROS levels. Measurements of the mitochondrial ROS levels were performed by a fluorescence spectrophotometer using DCFH-DA. Briefly, isolated mitochondria were incubated with a respiration buffer[39] and DCFH-DA was added (final concentration, 10 μM) to the mitochondrial samples and then incubated for 10 min. After entry, DCFH-DA was hydrolyzed to non-fluorescent dichloro-fluorescin (DCFH), which reacted with ROS and made highly fluorescent dichlorofluorescin (DCF). Then, the fluorescence intensity of DCF was quantified at time intervals of 5, 15, 30, 45 and 60 min using a Shimadzu RF5000U fluorescence spectrophotometer device at excitation and emission wavelengths of 500 nm and 520 nm, respectively.[40]

2.7.3. Measurement of the hippocampal mitochondrial membrane potentials. For the estimation of the mitochondrial membrane potentials (MMP), the mitochondrial uptake of a cationic fluorescent dye, Rhodamine 123 (Rh123), was used. Mitochondrial fractions in the MMP assay buffer were incubated with 10 μM of Rh123. Then, the fluorescence was monitored at

time intervals of 5, 15, 30, 45 and 60 min using the Shimadzu RF5000U fluorescence spectrophotometer device at an excitation wavelength of 490 and an emission wavelength of 535 nm.[41]

2.7.4. Measurement of hippocampal mitochondrial swelling. Determination of hippocampal mitochondrial swelling was done through changes in light scattering measured spectrophotometrically at 540 nm (30 °C).[42] The isolated brain mitochondria were suspended in swelling buffer and the absorbance was determined at 540 nm at time intervals of 5, 15, 30, 45 and 60 min with an ELISA reader apparatus (Tecan, Rainbow Thermo and Austria). A reduction in absorbance was an indicator of mitochondrial swelling.

2.7.5. Measurement of cytochrome *c* oxidase activity and assessment of outer mitochondrial membrane damage. Both mitochondrial outer membrane integrity and cytochrome *c* oxidase activity were measured using a cytochrome *c* oxidase assay kit (Sigma, St. Louis, MO). In the colorimetric trial, a decrease in absorbance was caused by oxidation of ferrocytochrome *c* at 550 nm to ferricytochrome *c* by cytochrome *c* oxidase. Experimental procedures were done according to the manufacturer's protocol; 20 μg of isolated mitochondrial fraction were used for each reaction, and duplicate reactions were performed for each assay. For the determination of total mitochondrial cytochrome-*c* oxidase activity, hippocampal mitochondrial fractions were diluted in the enzyme dilution buffer (10 mM Tris–HCl, pH = 7.0, containing 250 mM sucrose) with 1 mM *n*-dodecyl β-D-maltoside and placed on ice for 30 min. The reaction was performed by adding freshly ferrocytochrome-*c* substrate solution (0.22 mM) to the sample. The decrease in absorbance at 550 nm is linked to the oxidation of ferrocytochrome-*c* by cytochrome-*c* oxidase. Cytochrome-*c* oxidase activities were measured and normalized for the amount of protein per reaction and the results were shown as units per milligram of mitochondrial protein. The mitochondrial outer membrane integrity was determined by evaluating the cytochrome-*c* oxidase activity of the mitochondria in the presence or absence of *n*-dodecyl β-D-maltoside as a detergent. The mitochondrial outer membrane damage was measured through the ratio between cytochrome-*c* oxidase activity with and without detergent.

2.7.6. Measurement of mitochondrial lipid peroxidation. The MDA content was measured using the method of Zhang *et al.* 2008.[43] The mitochondrial fractions were incubated (1 h) with various concentrations of uranyl acetate at 30 °C; afterwards, 0.25 ml sulfuric acid (0.05 M) was added to 0.2 ml mitochondrial fractions, with the addition of 0.3 ml TBA 0.2%. All the microtubes were put in a bath of boiling water for 30 min. Finally, the tubes were placed in an ice-bath and 0.4 ml *n*-butanol was added to each of them. Then, they were centrifuged (3500 × *g*) for 10 min. The amount of MDA formed in each sample was evaluated by measuring the absorbance of the supernatant at 532 nm with an ELISA reader apparatus (Tecan, Rainbow Thermo, Austria). Standard tetramethoxypropane (TEP) was used and the MDA content was represented as μg mg^{-1} protein.[43]

2.7.7. Measurement of mitochondrial GSH contents. Mitochondrial GSH contents were evaluated using the spectrophotometer method and DTNB as the indicator for the isolated hippocampal mitochondria. The mitochondrial fractions were incubated with various concentrations of uranyl acetate (1 h) at 30 °C and then the mitochondrial fractions (0.1 ml) were added into phosphate buffers (0.1 mol L^{-1}) and DTNB (0.04%) in a total volume of 3.0 ml (pH 7.4). At 412 nm the yellow color was read on a spectrophotometer (UV-1601 PC, Shimadzu, Japan). The GSH amount was illustrated as μg mg^{-1} protein.[44]

2.7.8. Determination of mitochondrial ADP/ATP ratios. To determine the ADP/ATP ratios, the sample tissues were quickly homogenized (4 °C) in a 1 ml of ice cold TCA (6%) and then centrifuged at 12 000 g (10 min; at 4 °C). The supernatant was removed and neutralized with KOH (4 M). High-performance liquid chromatography (HPLC) was performed using a 510-pump and a solvent delivery system (Waters Chromatography Division, Milford, MA) and column (SUPELCOSIL1 LC-18-T, Supelco, Inc., Bellefonte, PA) equipped with a guard column, and finally 486 UV-Visible Detector (Waters Chromatography Division, Milford, MA). Isocratic elution (flow: 1 ml for 20 min at 254 nm) with tetra-butyl ammonium hydrogen sulfate (4 mM) in potassium phosphate buffer (0.1 M; pH 5.5) and methanol (85 : 15 v/v) was used according to the protocol provided.[45] The levels of ATP and ADP were measured by the standard curve and then the ratio was evaluated.

2.8. Hippocampal copper measurements

Copper concentrations were measured in the hippocampi using an atomic absorption spectrophotometry device (GBC Avanta Sigma; GBC Scientific Equipment PTY Ltd, Dandenong, Victoria, Australia). Briefly, a homogenized sample (0.5 g of wet weight) was digested with a mixture of nitric acid and perchloric acid (6 : 1, v/v) and the digested tissue was brought to a constant volume with double distilled deionized water.

2.9. Statistical analysis

The results for each group are presented as mean ± SD. GraphPad Prism-6 (GraphPad Software, La Jolla, CA) was used for the statistical analysis. A mean value for each dependent parameter of memory performance (traveled distance, escape latency and swimming speed) was evaluated over four trials in four training days. Mean values for each dependent measure of mitochondrial function (ROS level, MMP, and mitochondrial swelling) were also calculated over 5 time points (5, 15, 30, 45 and 60 min).

Statistical significance between the groups was determined by one-way analysis of variance (ANOVA) using a Bonferroni *post hoc* multiple comparison test and the *P* value was set lower than 0.05.

3. Results

3.1. Memory performance in MWM

Behavioral tests (MWM) were performed in order to evaluate the long term effects of copper exposure through the drinking water alone and in combination with Aβ (IH) on the learning and memory function of the rats. Fig. 1 shows the traveled distance (1A), escape latency (1B) and swimming speed (1C) for the control, vehicle (IH) and Aβ (IH) groups. Aβ caused a

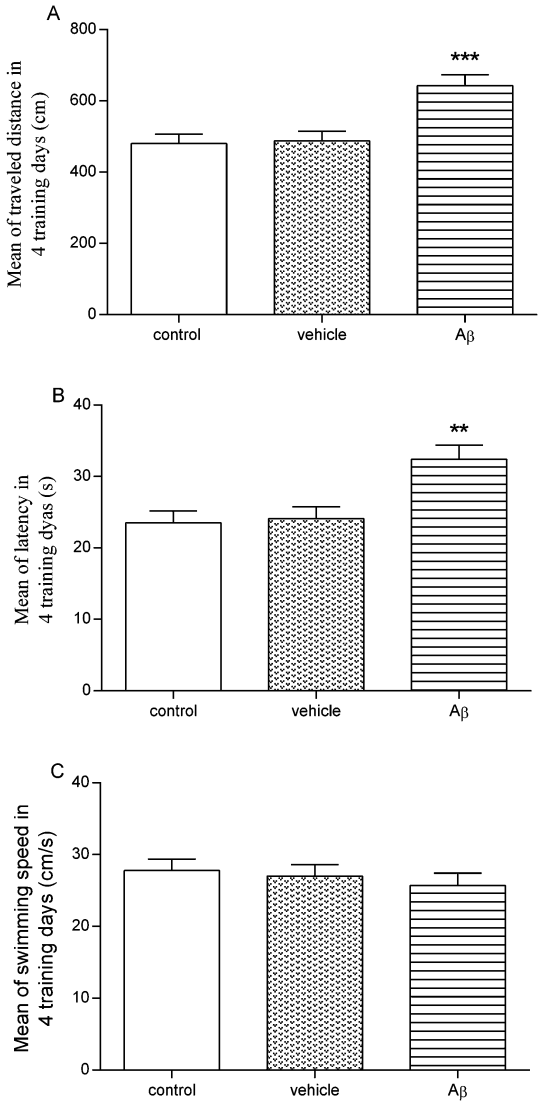

Fig. 1 A plot of the effect of the control, vehicle and Aβ groups on the means of traveled distance (A), escape latency (B) and swimming speed (C) over 4 days in the MWM test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group. Values represent means ± SEM of 7 animals per group.

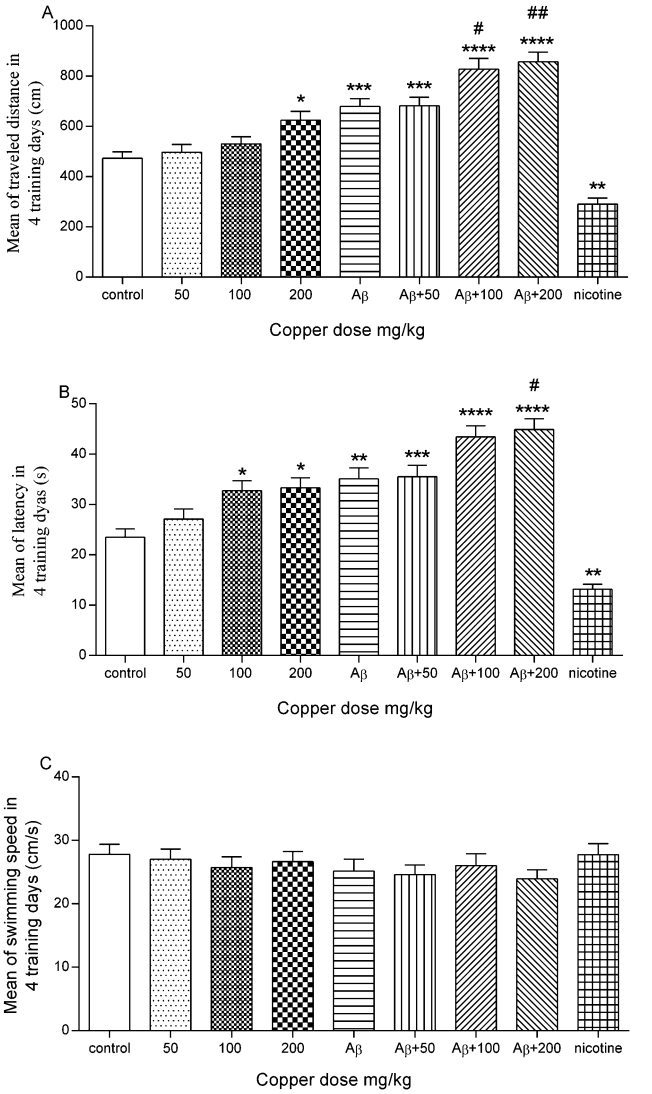

Fig. 2 A plot of the effect of copper doses, Aβ, copper doses + Aβ and nicotine treatment in animals on the means of traveled distance (A), escape latency (B) and swimming speed (C) over 4 days in the MWM test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ compared to control group. $\#P < 0.05$ and $\#\#P < 0.01$ compared to Aβ-injected group. Values represent means ± SEM of 7 animals per group.

significant increase in traveled distance ($P < 0.001$) and escape latency ($P < 0.01$) compared to the control group. In contrast to Aβ, sole vehicle administration did not significantly change the traveled distance and escape latency compared to the control group. As shown in Fig. 1C, no significant difference was observed between these groups in the swimming speed test.

Fig. 2 shows the results of nine groups, including the control group, copper groups (50, 100 and 200 mg kg^{-1} daily), Aβ (IH) injected group, combination groups that received Aβ (IH) plus copper (50, 100 and 200 mg kg^{-1} day^{-1}) and nicotine (IH) group.

Nicotine caused a significant improvement in the traveled distance and escape latency ($P < 0.01$) compared to the control group. Copper significantly increased the traveled distance (200 mg kg^{-1}: $P < 0.05$) and escape latency (100 and 200 mg kg^{-1}: $P < 0.05$) compared to the control group. In combination groups also, significant increases in the traveled distance (Cu 100 + Aβ: $P < 0.05$; Cu 200 + Aβ: $P < 0.01$) and escape latency (Cu 200 + Aβ: $P < 0.05$) compared to the group that had just received Aβ (Fig. 2A and B) were observed. It is noteworthy that no significant difference was observed in the mean of velocity between all nine groups on 4 training days of MWM (Fig. 2C).

The results of the probe trial test show that this parameter significantly decreased in the Aβ group ($P < 0.01$) compared to the control group. Also, there was no significant difference between the vehicle group and the control group (Fig. 3A). Copper doses (100 and 200 mg kg^{-1}: $P < 0.05$) significantly decreased the time spent in the target quadrant in the probe trial test compared to the control group. Moreover, a significant decrease for the Cu 200 mg kg^{-1} plus Aβ group ($P < 0.05$) compared to the Aβ injected group for this parameter was observed (Fig. 3B).

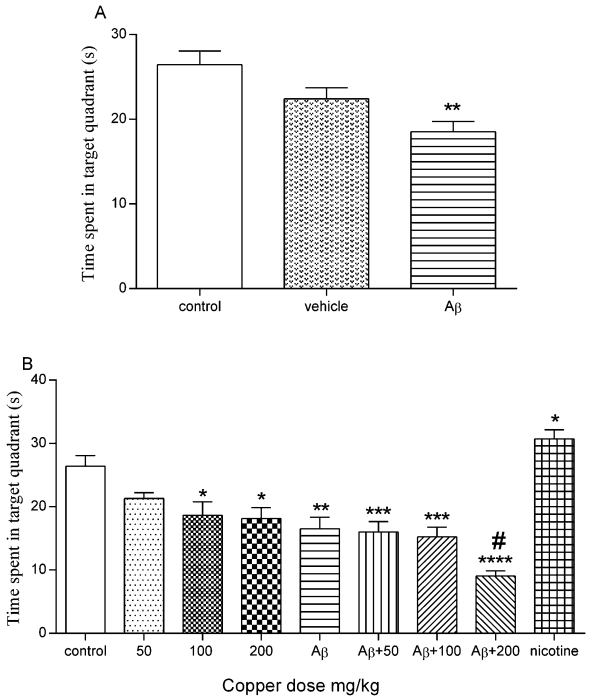

Fig. 3 A plot of the effect of the control, vehicle and Aβ groups (A) and copper doses, Aβ, copper doses + Aβ and nicotine groups (B) on time spent in the target quadrant in the probe trial test of MWM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to the control group. #$P < 0.05$ compared to Aβ-injected group. Values represent means ± SEM of 7 animals per group.

The results of these comparisons indicate that copper in the drinking water significantly increased the traveled distance and escape latency on four training days of MWM (Fig. 2A and B) and decreased the time spent in the target quadrant in the probe trial test of MWM (Fig. 3B), which illustrates that high doses of copper caused spatial memory impairment. Furthermore, the results show that copper in combination with Aβ could have intensified the increase in traveled distance and escape latency on the 4 training days of MWM (Fig. 2A and B) as well as reducing the time spent in the target quadrant in the probe trial test (Fig. 3B).

3.2. Mitochondrial function

3.2.1. Mitochondrial ROS formation. The results of the comparisons show that different doses of copper (100 and 200 mg kg^{-1}: $P < 0.05$) induced a significant increase in ROS generation in a concentration dependent manner, in comparison with the control group. In addition, a more substantial increase in mitochondrial ROS formation was observed in the Cu 200 mg kg^{-1} plus Aβ group ($P < 0.05$) compared to the Aβ peptide treated group (Fig. 4A).

3.2.2. Mitochondrial membrane potential. MMP is an important indicator of the mitochondrial inner membrane situation; therefore, the effect of copper on MMP was measured by Rh123 staining. As shown in Fig. 4B, Cu 100 mg kg^{-1} ($P < 0.05$) and 200 mg kg^{-1} ($P < 0.01$) strongly decreased MMP in a concentration-related manner, compared to the control group. Moreover, in the combination groups, significantly reduced MMP

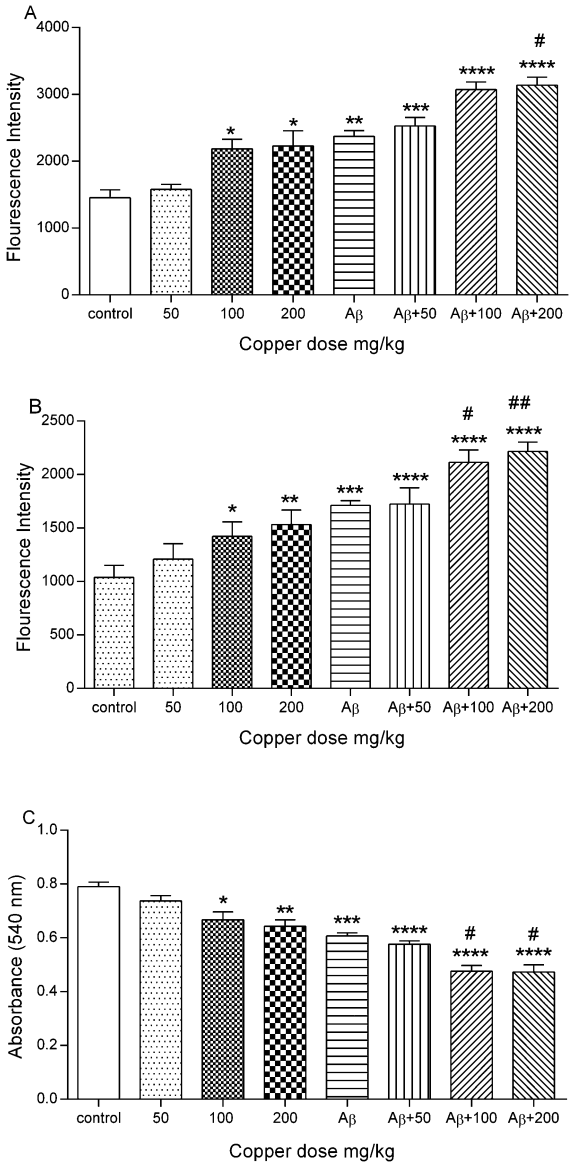

Fig. 4 A plot of the effect of the control, copper doses, Aβ and copper doses + Aβ groups on means of the ROS generation (A), MMP (B) and swelling (C) of rat hippocampal mitochondria at 5 time points (5, 15, 30, 45 and 60 min). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to control group. #$P < 0.05$ and ##$P < 0.01$ compared to Aβ-injected group. The values represent means ± SD (n = 7).

for the Cu 100 mg kg^{-1} + Aβ ($P < 0.05$) and Cu 200 mg kg^{-1} + Aβ groups ($P < 0.01$) compared with the Aβ treated group has been shown (Fig. 4B).

3.2.3. Mitochondrial swelling. Mitochondrial swelling is a determinant indicator of mitochondrial membrane permeability. The results of comparisons show that Cu 100 mg kg^{-1} ($P < 0.05$) and 200 mg kg^{-1} ($P < 0.01$) significantly caused mitochondrial swelling in a dose-dependent manner, in comparison with the control group (Fig. 4C). Furthermore, as illustrated in Fig. 4C, there was significant mitochondrial swelling for Cu 100 mg kg^{-1} + Aβ and Cu 200 mg kg^{-1} + Aβ ($P < 0.05$) compared to the Aβ-injected group.

3.2.4. Mitochondrial cytochrome *c* oxidase activity and outer membrane integrity. Cytochrome *c* oxidase activity (complex IV), a main enzyme in the mitochondrial respiratory complex, was also determined in the isolated Hippocampal mitochondria. As shown in Fig. 5A, there was a dose-dependent relationship between cytochrome *c* oxidase activity and Cu doses. Thus, significant collapses in the activity of this enzyme were observed in the Cu 100 and 200 mg kg^{-1} groups ($P < 0.05$) compared to the control group. Besides, combination groups also significantly decreased the enzyme's activity (for Cu 50 and 100 mg kg^{-1} plus Aβ: $P < 0.05$; for 200 mg kg^{-1} plus Aβ: $P < 0.01$) in comparison with the Aβ group (Fig. 5A). In mitochondrial outer membrane damage assays, the cytochrome *c* oxidase activity was measured in the presence/absence of the detergent, *n*-dodecyl β-D-maltoside. This ratio represents the percentage of hippocampal mitochondrial outer membrane damage. Our results show that copper significantly increased mitochondrial outer membrane damage in a concentration-dependent manner for the Cu 20 mg kg^{-1} group ($P < 0.05$) compared to the control group and for the Cu 200 mg kg^{-1} + Aβ group ($P < 0.05$) compared to the Aβ treated group (Fig. 5B).

3.2.5. Mitochondrial lipid peroxidation. The results show that lipid peroxidation (MDA formation) was increased by copper in a concentration dependent manner in the isolated hippocampal mitochondria. The amounts of MDA formation in the hippocampal mitochondria were 6.76 ± 1.2, 9 ± 1.2 and 9.2 ± 1.3 μg MDA per mg protein at 50, 100 and 200 mg kg^{-1} copper concentration, respectively, whereas that of the control group was 5.47 ± 0.9 μg MDA mg^{-1} protein. The MDA levels also increased to 10.02 ± 1, 10.6 ± 1.1, 13.3 ± 1.4 and 13.63 ± 1.4 (μg mg^{-1} protein) by Aβ, Cu 50 mg kg^{-1} + Aβ, Cu 100 mg kg^{-1} + Aβ and Cu 200 mg kg^{-1} + Aβ, respectively (Fig. 6A).

3.2.6. Mitochondrial glutathione content. Glutathione is a main antioxidant defense factor against various ROS, such as H_2O_2. GSH through a non-enzymatic reaction can directly react with H_2O_2 and is oxidized to GSSG.[46] Consequent to the observation of copper effect on H_2O_2 generation and lipid peroxidation, its possible effect on the antioxidant systems was also determined. The GSH concentration in the brain mitochondria was evaluated to determine the extent of oxidative stress induced by Cu. As shown in Fig. 6B, copper significantly decreased the GSH levels in a dose dependent manner. At 50, 100 and 200 mg kg^{-1} Cu concentrations, the GSH levels decreased to 26.13 ± 2.04, 21.17 ± 2.16 and 17.97 ± 2.45 (μg mg^{-1} protein), respectively, and for the control group it was 28.27 ± 1.9 μg mg^{-1} protein. The GSH levels also decreased to 15.7 ± 2, 14.73 ± 2.6, 10.77 ± 2.2 and 9.5 ± 2.8 μg mg^{-1} protein by Aβ, Cu 50 mg kg^{-1} + Aβ, Cu 100 mg kg^{-1} + Aβ and Cu 200 mg kg^{-1} + Aβ, respectively (Fig. 6B).

3.2.7. Mitochondrial ADP/ATP ratios. Mitochondrial respiratory processes are required for ATP production in

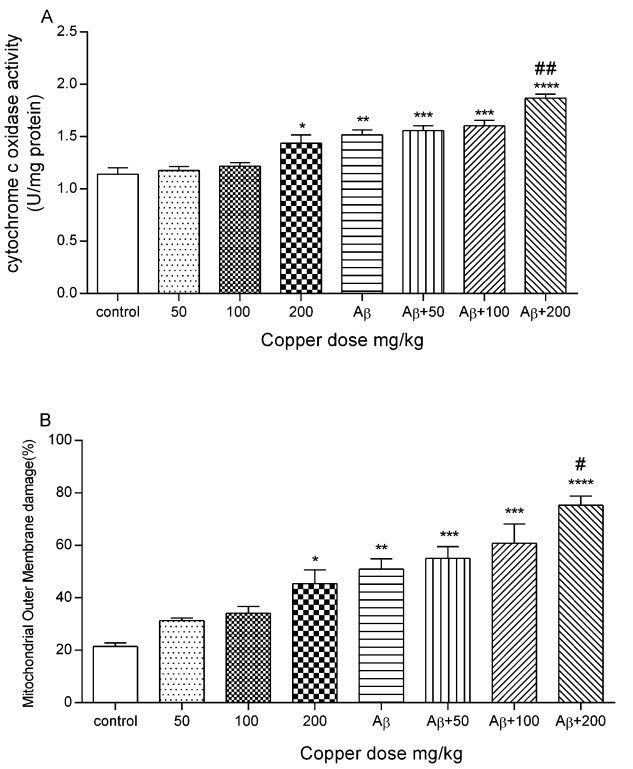

Fig. 5 A plot of the effect of the control, copper doses, Aβ and copper doses + Aβ groups on cytochrome *c* oxidase activity (A) and mitochondrial outer membrane damage (B). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to control group. #$P < 0.05$ and ##$P < 0.01$ compared to Aβ-injected group. The values represent means ± SD (n = 7).

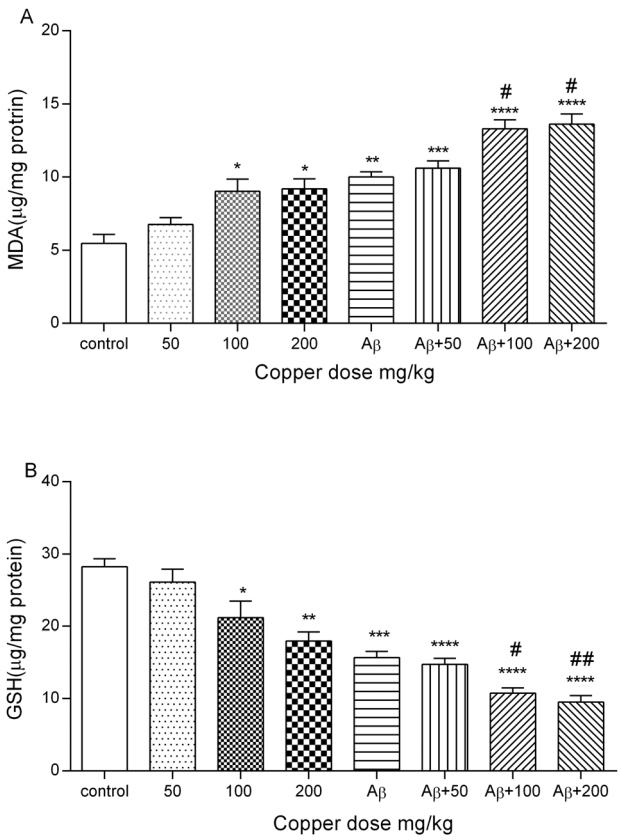

Fig. 6 A plot of the effect of the control, copper doses, Aβ and copper doses + Aβ groups on lipid peroxidation (A) and GSH content (B). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to control group. #$P < 0.05$ and ##$P < 0.01$ compared to Aβ-injected group. The values represent means ± SD (n = 7).

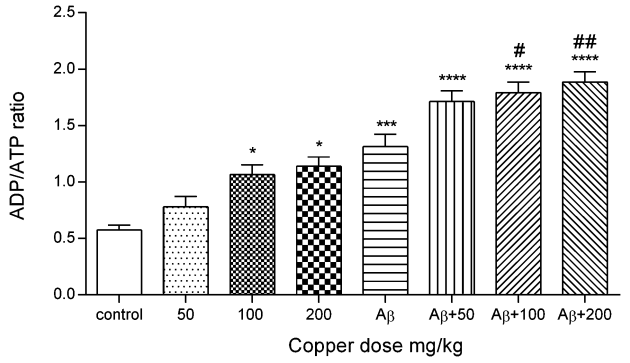

Fig. 7 A plot of the effect of the control, copper doses, Aβ and copper doses + Aβ groups on hippocampus ADP/ATP ratios. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to control group. #$P < 0.05$ and ##$P < 0.01$ compared to Aβ-injected group. The values represent means ± SD ($n = 7$).

Table 1 Brain hippocampal copper concentrations after 3 weeks' exposure to different doses of Cu in the drinking water

Groups	Hippocampal copper concentration (μg g^{-1} wet weight)
Control	0.19 ± 0.02
Cu 50 mg kg^{-1}	0.21 ± 0.03
Cu 100 mg kg^{-1}	0.25 ± 0.03**
Cu 200 mg kg^{-1}	0.27 ± 0.04***
Aβ 1 μg μl^{-1} per side	0.2 ± 0.03
Aβ + Cu 50 mg kg^{-1}	0.22 ± 0.02
Aβ + Cu 100 mg kg^{-1}	0.26 ± 0.04**
Aβ + Cu 200 mg kg^{-1}	0.27 ± 0.03***

The copper concentration was determined by atomic absorption spectrophotometry as described in the materials and methods. **$P < 0.01$, ***$P < 0.001$ compared with the control group. The values represent means ± SD for seven rats in each group.

mitochondria, since copper impaired the mitochondrial respiration; therefore, we measured the ADP/ATP ratios in isolated hippocampal mitochondria. As shown in Fig. 7, there was a statistically significant increase in the ADP/ATP ratios in the Cu 100 and 200 mg kg^{-1} groups ($P < 0.05$) compared to the control group. Furthermore, a significant increase in the ADP/ATP ratio in the Cu 100 mg kg^{-1} + Aβ ($P < 0.05$) and Cu 200 mg kg^{-1} + Aβ groups ($P < 0.01$) compared to the Aβ treated group was observed (Fig. 7).

3.3. Brain hippocampal copper concentrations

The increase in hippocampal Cu concentrations was proportional to the dose of exposure. The Cu concentrations in the hippocampi of the rats were significantly higher in the groups receiving copper 100 mg kg^{-1} and 100 mg kg^{-1} + Aβ ($P < 0.01$) and also in the groups receiving Cu 200 mg kg^{-1} and 200 mg kg^{-1} + Aβ ($P < 0.001$) compared to the control group (Table 1).

4. Discussion

The results of this study show that chronic copper sulfate administration (for 3 weeks) in the drinking water markedly induced learning and memory impairment. Sole copper sulfate administration in rats induced oxidative damage in the hippocampal mitochondria. In co-administration with amyloid beta, copper sulfate could potentiate Aβ induced learning and memory impairment and mitochondrial dysfunction in a concentration dependent manner. Squitti *et al.*[47] studied the amount of serum copper in 79 Alzheimer's disease patients (54 women and 25 men; mean age 74.3 years) and in 76 individuals with normal cognitive function (43 women and 33 men; mean age 70.1 years). Their studies showed that the copper content was significantly higher in AD patients compared to the control groups and also an increase of 1 μmol L^{-1} in the serum copper level could elevate the risk of having AD by about 80% and has a close relationship with poor neuropsychological performance and atrophy of the medial temporal lobe. To the best of our knowledge, this is the first study that reports the effects of copper alone and with amyloid beta on spatial memory and mitochondrial impairments. This finding supports previous research, which found accumulation of amyloid beta peptides and memory deficit caused by copper neurotoxicity. Sparks *et al.*[48] reported that adding 0.12 PPM copper sulfate (0.75 μM) for ten weeks to the drinking water of cholesterol-fed beagles, spontaneously hypercholesterolemic Watanabe rabbits and PS1/APP transgenic mice generated significantly elevated brain levels of Aβ. Becaria *et al.*[49] also showed that 3 months' exposure of mice to copper sulfate (8 μM) in the drinking water initiated oxidative and inflammatory events in the brain and that it may function to enhance APP levels with aluminum co-exposure. Ceccom *et al.*[50] reported the capacity of a new copper-specific chelating agent, a bis-8-aminoquinoline PA1637, to entirely reverse the deficit of episodic memory after 21 days of treatment by the oral route on non-transgenic amyloid-impaired mice. Kumar *et al.*[51] declared that all behavioral tests (percentage attention score in Y-maze, maximum peak force in grip strength and latency to fall in rotarod) were significantly decreased in rats that received oral copper sulfate in doses of 100 and 200 mg kg^{-1} (especially in the 200 mg kg^{-1} group) at all time points (30 days, 60 days and 90 days of exposure) compared to the control group. However, the findings of our study differ from the study by Leiva *et al.*[52] which suggests no difference between the copper-treated and control groups in the training days of MWM. A possible explanation for this difference might be the lower dose of Cu in this study compared to our study. In the study by Leiva *et al.*,[52] $CuSO_4$ dissolved in saline was injected (ip) 1 mg kg^{-1} for 30 days and then MWM was performed. Singh *et al.*[53] elucidated the mechanism of Cu-induced Aβ accumulation in the brains of mice and their studies showed that in aging mice, accumulation of Cu in the brain capillaries was associated with its decrease in low-density lipoprotein receptor-related protein 1 (LRP1), an Aβ transporter, and excess brain Aβ levels. These effects were reproduced by chronic dosing with low levels of Cu through drinking water and they also reported that Cu not only down-regulated LRP1 in the brain capillaries but also increased Aβ generation and neuroinflammation. One of the possible mechanisms of copper induced neurotoxicity in various brain systems is mitochondrial function impairment, which has not been studied in any of the previous studies in relation to learning and memory deficit.

It has been known that in copper overload disorders, the mitochondrion is the main target of toxicity, which is associated with mitochondrial ETC dysfunction and oxidative stress-mediated reduction of the mitochondrial membrane potential (Ψ_m), mitochondrial membrane integrity and ATP production.[54]

The results of this study demonstrate that Cu in high concentrations could increase and potentiate (in Aβ treated animals) ROS generation in hippocampal mitochondria and cause memory disorder. This finding is in agreement with the findings of White *et al.*,[55] which showed that in primary mouse neuronal cultures, homocysteine, a thiol-containing amino acid, in the presence of micromolar concentrations of Cu(II) produced high levels of hydrogen peroxide and promoted Aβ/Cu-mediated hydrogen peroxide generation and neurotoxicity.

They also suggested that high levels of copper and/or homocysteine in the elderly could promote serious oxidative damage to neurons and may indicate additional risk factor pathways which conspire to generate AD or related neurodegenerative diseases. Changing or inhibiting the activity of mitochondrial complexes leads to MMP disruption, which affects the generation rate of ATP *via* complex V. This study indicates that following Cu administration, MMP was reduced in a dose dependant manner and also, in elevated concentrations, Cu could exacerbate MMP reduction in the Aβ treated groups. Ozcelik *et al.*[5] reported that Cu overload in the brain reduced SOD activities and GSH levels and also increased MDA levels due to lipid peroxidation. Glutathione (GSH) is one of the main non-enzymatic antioxidant systems against hydrogen peroxide and other ROS, which constitutes almost 10–15% of total cellular GSH in mitochondria.[56] Therefore, the decrease of reduced glutathione content in mitochondria could cause severe weakness in their defense system against oxidative damage and a further rise in lipid peroxidation. As our data show, copper in a dose dependent manner induced and exacerbated (in Aβ-injected rats) the GSH oxidation and lipid peroxidation in exposed hippocampal mitochondria. Moreover, GSH is required for the maintenance of thiol groups in mitochondrial membrane proteins in the reduced form.[43] When these thiol groups are oxidized, conformational changes arise in the pore complex that lead to mitochondrial permeability transition (MPT).[57] Thus, Cu-induced ROS production not only leads to GSH oxidation and lipid peroxidation, it could also destroy the mitochondrial membrane integrity and open the MPT pores. The opening of MPT pores initiates the onset of apoptosis and necrosis mechanisms.[58] Mitochondrial swelling is a structural change of isolated mitochondria undergoing permeability transition.[59] Several agents such as oxidants, heavy metals and sulfhydryl reactive compounds are capable of inducing the permeability transition.[59] In this study, copper in a dose dependent manner was found to cause and worsen (in Aβ-injected rats) mitochondrial swelling in the brain hippocampus. It is possible that mitochondrial swelling occurs due to the destruction of mitochondrial membrane integrity resulting from elevated ROS production, which causes the collapse of MMP. The present finding seems to be consistent with other research, which showed copper induced mitochondrial swelling *in vitro*.[60] This study shows that copper in a dose dependent manner significantly increased and deteriorated (in Aβ-injected rats) the outer membrane damage. As a result, Cu-induced lipid oxidation in hippocampal mitochondria (as a consequence of respiratory chain impairment and a decrease in GSH) could promote mitochondrial membrane destruction and finally the lack of mitochondrial outer membrane integrity. Mitochondria are essential for cell survival due to their roles as moderators of apoptosis.[61] Programmed cell death may occur when the amount of ROS produced in the mitochondria cannot be neutralized by the radical-scavenging cellular antioxidants. The mechanism of ROS-mediated apoptosis illustrates the involvement of the MPT pore opening and release of cytochrome *c* from mitochondria and their ATP-dependent interaction with cytosolic factors for the formation of apoptosome in order to activate caspase-9 and caspase-3, respectively.[62]

Crouch *et al.*[63] indicated that Aβ 1–42, in its dimeric conformation is associated with brain mitochondrial function and the presence of Cu^{2+} is a potent inhibitor of cytochrome *c* oxidase (COX) activity in a dose-dependent manner. They concluded that inhibition of COX activity by Cu^{2+}-dependent Aβ may be a main contributor to the neurodegenerative process in Alzheimer's disease. We also investigated the effect of copper on the activity of complex IV (cytochrome *c* oxidase) of the mitochondrial electron transfer chain. As shown in the results, copper in a concentration dependent manner significantly decreased cytochrome *c* oxidase activity in the mitochondria of brain hippocampi and also could exacerbate the reduced activity of cytochrome *c* oxidase in Aβ-injected rats.

The most important mitochondrial function is ATP production through oxidative phosphorylation. Furthermore, the ATP level defines the process of cell death in target cells.[64] In fact, ATP acts as a switch between apoptosis and necrosis. Apoptosis requires ATP, while depletion of ATP shift cells to necrosis and interrupts apoptosis.[64] We found that copper in a concentration dependent manner decreased ATP production and increased the ADP/ATP ratio in isolated hippocampal mitochondria and could also intensify the increased ADP/ATP ratio in Aβ treated rats. This might be due to the inhibition of the mitochondrial respiratory chain and MPT pore opening. Pereira *et al.*[65] reported that inhibition of the mitochondrial ETC could lead to a reduction in MMP and ATP production. In fact, when the MPT pore opened, unlimited protons moved across the inner membrane and subsequently resulted in the uncoupling of oxidative phosphorylation.[61] The remaining ATP was consumed immediately for MMP maintenance, resulting in a further decrease in the ATP level; this leads to exacerbation of ROS generation.[61] Hosseini *et al.*[60] suggested that mitochondrial toxicity of Cu^{2+} on isolated rat liver could lead to increased disturbance in oxidative phosphorylation and a decreased ATP/ADP ratio. They also showed that Cu^{2+} induced liver toxicity in a concentration and time-dependent manner because of its effects on liver hepatocyte mitochondria through lipid peroxidation, ROS formation, release of cytochrome *c* and mitochondrial membrane potential reduction, which start cell death signalling.

5. Conclusion

The results of this study show that chronic copper (for 3 weeks) in drinking water results in increased ROS formation followed

by MMP collapse, increased ADP/ATP ratio, mitochondrial outer membrane damage, mitochondrial swelling and finally decreased cytochrome *c* oxidase activity in a concentration dependent manner. Given the key role of mitochondria in synaptic plasticity as the important component in learning and memory formation, the copper-induced dysfunction of neuronal mitochondria could be considered as the mechanism of copper-induced spatial learning and memory impairments in rats. Copper also could exacerbate the destructive effects of amyloid beta on brain hippocampal mitochondria and spatial memory in Aβ-injected rats.

Abbreviations

MWM	Morris water maze test
Aβ	Beta-amyloid peptide 1–42
ROS	Reactive oxygen species
MMP	Mitochondrial membrane potential
IH	Intrahippocampally.

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